Gene therapy: adenovirus vectors

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The past year has seen a proliferation in the use of recombinant, replication-defective adenoviruses for experimental models of gene therapy. The fact that adenovirus infects most cell types with no requirement for cell division, combined with the high titers and high efficiency of gene transfer obtainable with recombinant adenovirus, make it a promising system for *in vivo* human gene therapy.

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Introduction

Gene therapy represents a novel approach for treating diseases that is based on the directed modification of gene expression in somatic cells of the patient. Early paradigms of gene therapy have focused on the transplantation of autologous cells that are genetically modified *ex vivo* with recombinant retroviruses. Several clinical protocols of *ex vivo* gene therapy have been initiated, including the transplantation of adenosine deaminase-transduced lymphocytes for the treatment of severe combined immunodeficiency and the transplantation of low density lipoprotein (LDL) receptor-transduced hepatocytes for the treatment of familial hypercholesterolemia (for a review, see [1]). It is clear that *ex vivo* gene therapy is useful for treating certain lethal diseases such as those described above. However, a broader application of this technology will require less invasive, more practical approaches in which the therapeutic gene is directly targeted to appropriate cells *in vivo*.

Previous attempts to directly target genes to somatic cells *in vivo* have made use of a variety of viral and synthetic approaches. Examples include retroviruses [2] and DNA–protein complexes for hepatocyte gene transfer [3,4], liposomes for gene transfer to airway epithelial cells [5], retroviruses and liposomes for endothelial cell gene transfer [6,7], and naked DNA for gene transfer to skeletal and cardiac myocytes [8–10]. The therapeutic potential of most of these approaches is limited because of the lack of specificity and extremely low efficiency of gene transfer. The development of recombinant adenoviruses for gene transfer has suggested new strategies for *in vivo* gene therapy. In this review we summarize recent studies on recombinant adenoviruses for *in vivo* gene therapy.

Adenoviruses

Adenovirus (Ad) type 2 and type 5 (Ad2 and Ad5, respectively), which cause respiratory disease in humans, are currently being developed for use in gene therapy; both belong to a subclass of adenovirus that are not associated with human malignancies [11]. In further support of the safety of recombinant adenoviruses for gene therapy is the long-standing extensive and effective use of live adenovirus vaccines in human populations [11,12]. The adenovirus genome is composed of linear, double-stranded DNA of approximately 36 kb in length, which is divided into 100 map units (mu), each of which is 360 bp in length. The DNA contains short inverted terminal repeats (ITRs) at each end of the genome that are required for viral DNA replication. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions, based on expression before or after the initiation of viral DNA synthesis. Adenoviruses have a lytic life cycle; they are taken up by cells through as yet unidentified receptors, enter the endosome, and from there the virus enters the cytoplasm and begins to lose its protein coat. The viral DNA migrates to the nucleus, where it retains its linear structure rather than integrating into the chromosome.

The recombinant, replication-deficient adenoviruses that have been developed for gene therapy contain deletions of the entire E1a and part of the E1b regions (for a review, see [13]). The E1 region regulates adenoviral transcription and is required for viral replication. The replication-defective virus is grown on an adenovirus-transformed human embryonic kidney cell line, the 293 cell, which contains the E1 region of adenovirus. E1-deleted viruses are capable of replicating and producing infectious virus in the 293 cells, which provide the E1a- and E1b-region gene products *in trans*. The result-

Abbreviations

α_1AT—α_1 antitrypsin; CF—cystic fibrosis; CFTR—CF transmembrane conductance receptor; CMV—cytomegalovirus; ITR—inverted terminal repeat; LDL—low density lipoprotein; MLP—major late promoter; mu—map unit; OTC—ornithine transcarbamylase.

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EXHIBIT B

ing virus is capable of infecting many cell types and can express the introduced gene (providing it carries its own promoter) but cannot replicate in a cell that does not express the E1 functions unless the cell is infected at a very high multiplicity of infection.

A scheme for constructing a recombinant adenovirus is depicted in Fig. 1. Because of the size of the adenovirus genome (and the resulting high frequency of restriction-enzyme sites), it is difficult to directly clone recombinant genes into the adenovirus genome. Instead, homologous recombination is used. A plasmid is constructed which contains the transgene, such as *lacZ*, under the control of the cytomegalovirus (CMV) promoter, or another promoter of choice. The recombinant minigene is flanked on the 5′ border with ITR sequences (0–1 mu) and on the 3′ border with approximately 2 kb of adenoviral sequences (9–16 mu) that are the target for homologous recombination. The linearized plasmid is co-transfected with wild-type Ad5 DNA that has been digested with a restriction enzyme to remove the left end of the adenovirus (0–2 mu). This renders the wild-type DNA non-infectious. Only products of homologous recombination should produce infectious virus following transfection into 293 cells. The recombinant virus is purified by at least two rounds of plaque purification.

Lung

Because of their natural tropism for the respiratory tract, adenoviruses have been considered as candidates for gene therapy of the two most common hereditary lung diseases, cystic fibrosis (CF) and α_1-antitrypsin (α_1AT) deficiency. Rosenfeld *et al.* [14] chose the cotton rat lung as an animal model for the delivery of recombinant adenoviruses because the cotton rat has been shown to display a sensitivity to infection with adenovirus similar to that seen in humans. Tracheal instillation of recombinant adenovirus encoding α_1AT resulted in cells expressing α_1AT, as determined by *in situ* hybridization and by detection of α_1AT in lavage fluid. The *CFPAC* cell line (a pancreatic acinar cell line isolated from a CF individual) was used to demonstrate that cells infected with recom-

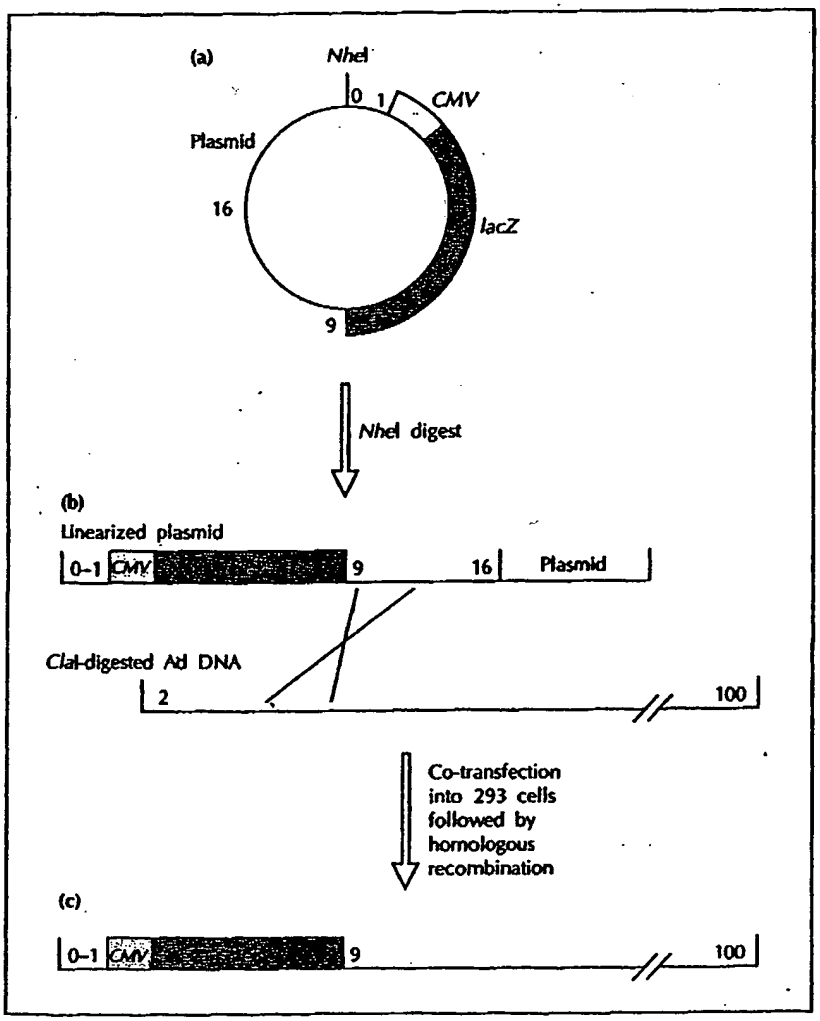

Fig. 1. Generation of recombinant adenoviruses. **(a)** A plasmid containing a transgene (for example, the β-galactosidase gene, *lacZ*, under control of the cytomegalovirus (CMV) promoter is constructed. The plasmid sequences indicated incorporate the ampicillin resistance gene and bacterial origin of replication. **(b)** Linearized plasmid DNA (here using restriction enzyme *NheI*) and *ClaI*-digested adenovirus (Ad) DNA (lacking the left end of the adenovirus, which renders it non-infectious) are co-transfected into 293 cells. **(c)** As a result of homologous recombination (within 9–16 map units) indicated by the large cross, the recombinant E1a/partial E1b-deleted adenovirus containing the *lacZ* gene is produced. (The recombinant virus is infectious in the 293 cells, which provide the E1-region gene products *in trans*.) The numbers indicate map units of the adenovirus and the diagonal lines represent a length of DNA not shown. See text for further details.

binant adenovirus encoding human CF transmembrane conductance receptor (CFTR), Ad-CFTR, were capable of significant chloride transport in response to forskolin [15••], a hallmark of CFTR-mediated chloride transport. Furthermore, human CFTR transcripts and protein were detectable in the lungs of cotton rats following tracheal instillation of Ad-CFTR. All major cell types of the cotton rat lung airway epithelium are capable of being infected by adenovirus, suggesting that adenovirus may be an efficient system for obtaining gene expression in all cells of the airway epithelium [16]. The availability of a mouse model for CF will be valuable in evaluating the efficacy of *in vivo* gene therapy with recombinant adenoviruses [17].

As a model for studying the infectibility of human airway epithelial cells *in vivo*, a xenograft system has been developed [18]. Denuded rat trachea are seeded with freshly isolated human bronchial epithelial cells and implanted into nude mice. Within three weeks, the epithelium showed an organization and distribution of cell types similar to that found in the native airway [18]. Adenoviruses encoding *lacZ* or *CFTR* were then introduced into the xenografts, which were analyzed for gene expression for up to five weeks post-infection [19••]. On average, 11% of cells expressed the transgene, and expression remained stable over the course of the experiment. All cell types, with the exception of basal cells, were infected with the recombinant adenoviruses in proportion to their distribution. In contrast, when retroviruses were used in the xenograft model, gene transfer was achieved only when the epithelial cells were still actively dividing and had not yet formed the fully differentiated epithelium at the time of exposure to virus [18].

The adenovirus-infected human xenografts were also assayed for the expression of several adenovirus-encoded genes. A small percentage of cells expressed an early gene product (the E2a-encoded DNA-binding protein), but the hexon and fiber proteins (late gene products) were not detectable [19••]. This suggests that adenovirus is not completing its replicative cycle, but the expression of the E2a gene product also indicates that the E1 deletion in the recombinant adenoviruses is not sufficient to eliminate all adenoviral gene expression.

Liver

Stratford-Perricaudet *et al.* [20] introduced a recombinant adenovirus encoding the rat ornithine transcarbamylase (*OTC*) gene under the control of the adenoviral major late promoter (MLP) intravenously into Spf-ash mice, a strain that expresses only low levels of OTC. In approximately 25% of these animals, hepatic OTC reached normal levels up to two months post-injection. One animal, allowed to survive long-term, showed partial reversal of orotic acidura through 13 months post-injection, and viral OTC transcripts could be detected in the liver at 15 months post-injection. This suggests that hepatic delivery of recombinant adenovirus can be achieved through intravenous injection, and that it is possible to achieve long-term gene transfer.

Recombinant adenoviruses have also been shown to be capable of infecting rat hepatocytes both *in vitro* and *in vivo* [21]. Rat livers were infected with adenoviruses encoding either β-galactosidase or human α_1AT by infusion of the virus into the portal vein. Three days post-infusion, the liver exposed to the *lacZ* virus contained hepatocytes expressing β-galactosidase activity at a frequency of approximately 1%. Expression of β-galactosidase was not detected in other organs, such as lung, spleen, muscle, kidney, brain and testis. Infusion of adenovirus encoding α_1AT resulted in detectable serum levels of human α_1AT up to four weeks post-infusion.

Central nervous system

Recombinant adenovirus was considered a possible candidate for gene therapy in the brain because neurons have a limited capacity for replication. With other vectors such as retrovirus, which require host DNA replication for infection, gene transfer has only been possible in fetal or neonatal brain cells [22,23]. Several studies have shown that adenovirus can be used to infect brain cells *in vivo*. When a recombinant, replication-defective adenovirus encoding *lacZ* was introduced into the caudate putamen of mice, a variety of different cell types were infected, including neurons, oligodendrocytes, and myelinated axons [24••]. There was no accompanying morbidity, nor was there any evidence for virus replication or β-galactosidase expression beyond the brain parenchyma. Similarly, following introduction of recombinant adenovirus into the rat hippocampus and substantia nigra, β-galactosidase expression was detected in microglial cells, astrocytes, and neurons [25]. In both studies, expression of β-galactosidase persisted through at least eight weeks, although the number of *lacZ*-positive cells decreased. Two additional studies support these results: in one, the *lacZ*-encoding adenovirus was injected into specific areas of the rat brain with no apparent pathogenicity except where extremely high titers of adenovirus were used [26]; in the second, introduction of an adenovirus encoding human α_1AT into the lateral ventricle of rats resulted in α_1AT secretion into the cerebral spinal fluid for up to one week [27]. These studies demonstrate that it should be possible to express a transgene in the brain to achieve a biological effect; namely, by the expression of neurotransmitters or growth factors.

Endothelial cells

A further target for gene therapy is endothelial cells, specifically because they are in direct contact with the bloodstream, allowing for the direct secretion of proteins into the circulatory system. One application of gene delivery to endothelial cells would be to prevent local atherosclerotic plaque formation. The feasibility of this

approach was demonstrated in experiments in which endothelial cells were isolated from human umbilical vein, and infected *in vitro* with adenoviruses encoding either *lacZ* or α_1AT, as marker genes for intracellular proteins and secreted proteins, respectively [28]. *LacZ* expression could be obtained in up to 100% of the cultured cells, and in parallel, secretion of α_1AT persisted for at least 14 days. To more closely mimic the *in vivo* situation, intact umbilical veins were directly exposed to the recombinant adenoviruses. Expression of β-galactosidase occurred at a high level, with no apparent alterations in the morphology of the infected endothelial cells. Infection with the α_1AT adenovirus resulted in synthesis and secretion of α_1AT. These results strongly suggest that *in vivo* gene therapy of endothelial cells should be possible.

Muscle

Intravenous injection of recombinant adenovirus into mice leads to transgene expression in many organs, including liver, lung, intestine, heart, and skeletal muscle [29]. Adenovirus-encoded *lacZ* expression in muscle tissue persisted through 12 months post-injection, although the proportion of positive cells decreased. The length of expression may be due to the fact that myotubes have a very slow rate of turnover compared with other cells. Injection of neonatal mice with recombinant adenovirus yielded a higher level of infection than in adult mice. Intramuscular injection [30] resulted in a very limited region of cells expressing β-galactosidase activity as compared with the intravenous route of delivery. Southern-blot analysis showed that all of the detectable adenovirus DNA remained extrachromosomal [29]. A recombinant adenovirus has been generated that encodes the human minidystrophin gene [31••], as the full-length cDNA is too large to be used in the present adenovirus system. Injection of this recombinant adenovirus directly into the biceps femoris of a mouse model of Duchenne muscular dystrophy, the *mdx* mouse, resulted in expression of the minidystrophin gene in 5–50% of muscle fibers. Expression appeared to be stable for at least three months [31••]. These results point the way to using recombinant adenovirus encoding dystrophin as a form of therapy for Duchenne muscular dystrophy.

Conclusion

The ability to infect numerous different cell types and the absence of a requirement for dividing cells make adenovirus an attractive candidate for *in vivo* gene therapy. In addition, as adenovirus appears not to integrate into the host cell genome, the potential threat of insertional mutagenesis is greatly reduced. However, that same feature may also result in a lack of persistence of the adenoviral DNA, and thus repeated administrations of adenovirus may be required. While this may be feasible, the generation of an immune response to the infused adenovirus has the potential to prevent repeated infections. This remains to be evaluated in the *in vivo* situation. In addition, despite the E1 deletions in adenovirus, the possibility remains that the recombinant virus retains the capacity to replicate at very low levels. The potential effects of such replication, and of adenoviral gene expression in the presence or absence of replication remain to be determined. The generation of adenoviral vectors with further deletions or mutations in additional adenovirus genes may eliminate such drawbacks. Meanwhile, the current state of adenovirus recombinant technology is rapidly making possible the use of *in vivo* gene therapy in animal models. Given the high levels of expression obtainable with recombinant adenovirus, significant biological effects can be achieved, although such effects may be transient.

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